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GRANT NUMBER: DAMD17-94-J-4247

TITLE: Genetic Analysis of Human Breast Cancer

PRINCIPAL INVESTIGATOR: Doctor Michael H. Wigler

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

REPORT DATE: August 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1996	Annual (15 Jul 95 - 14 Jul 96)

4. TITLE AND SUBTITLE
Genetic Analysis of Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4247

6. AUTHOR(S)
Doctor Michael H. Wigler

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimit

12b. DISTRIBUTION

13. ABSTRACT *(Maximum 200*

Cancer is a disorder brought upon by the accumulation of specific mutations in the cancerous cells. Our understanding of the disease, and potentially its diagnosis and therapeutic treatment, is enhanced by defining the genetic lesions that cause it. Representational difference analysis, or RDA, was developed to do just this. RDA is a DNA subtraction methodology that finds sequences present in one DNA population, the tester, that is absent or reduced in a second, the driver (1, 2). RDA has been used to discover sequences lost or amplified in the genomic DNA of the cancerous cells (3). Genetic loss and gene amplification are hallmarks of tumor suppressor genes and oncogenes, respectively. We have been applying RDA to the discovery of sequences lost in breast cancer. At least a dozen loci undergoing loss in breast cancer have been identified. The transcriptional potential of these loci is being explored. Characterization of many other RDA probes is in progress. We expect that the continued execution of our stated plan will accomplish our stated goal, the identification of tumor suppressor genes that are commonly involved in breast cancer.

14. SUBJECT TERMS Breast cancer	15. NUMBER OF PAGES 8
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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Michael H W, Jr Aug. 13, 1996

PI - Signature Date

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INTRODUCTION

Cancer is a disorder brought upon by the accumulation of specific mutations in the cancerous cells. Our understanding of the disease, and potentially its diagnosis and therapeutic treatment, is enhanced by defining the genetic lesions that cause it. Representational difference analysis, or RDA, was developed to do just this. RDA is a DNA subtraction methodology that finds sequences present in one DNA population, the tester, that is absent or reduced in a second, the driver (1, 2). To do this we make representations, called amplicons, of the genomes we wish to compare. An amplicon consists of the small amplifiable fragments that result after a genome is digested with a given restriction endonuclease. RDA has been used to discover sequences lost or amplified in the genomic DNA of the cancerous cells (3). Genetic loss and gene amplification are hallmarks of tumor suppressor genes and oncogenes, respectively. We have been applying RDA to the discovery of sequences lost in breast cancer.

RESULTS

The application of RDA to cancer requires the availability of matching tumor and normal DNA from the same individual, as otherwise the cloning of DNA polymorphisms results. The vast majority of available tumor material is not provided with accompanying normal cell samples. However, all tumors contain normal stroma. Since many tumors are aneuploid, we have chosen to apply RDA to tumor biopsies that can be sorted by flow cytometry into aneuploid (tumor) and diploid (normal) nuclei. Our studies have confirmed the utility of samples prepared in this way (3).

To date a total of about 550 human breast cancer biopsy samples have been obtained from collaborating hospitals. These include about 200 from Sloan-Kettering Memorial Hospital, about 150 from the Cooperative Human Tissue Network, about 150 from North Shore University Hospital, and a smattering from local medical centers. DNA content analysis by flow cytometric techniques have been performed on all of these samples. Of the samples analyzed nearly 200 have been sorted into diploid and aneuploid fractions. DNA has been prepared for RDA from some of these sorted fractions.

Using DNA samples isolated from aneuploid (tumor) nuclei and diploid (normal) nuclei, fractionated by fluorescence-activated cell sorter from breast cancer biopsies, about twenty pairs of normal and tumor DNAs have been subjected to RDA. Many candidate probes have been isolated and we are at various stages of analysing these.

The results for probes mapping to 3p21 were described in last year's report. This locus is identical to the FHIT locus identified by Ohta and coworkers (4). We find that this region can undergo rearrangement without perturbing the structure of the transcript they describe (5). We are employing techniques of exon tropping (6) to

identify other candidate transcripts from this region, and will test these for functional loss coinciding with genetic rearrangements. Similar work on the 20p11 locus, described last year, has so far failed to reveal bonafide transcripts altered in cancer. Both the 3p21 and 20p11 loci were identified by examination of gastrointestional cancers, but are also rearranged in some breast cancers.

Most of our effort has gone into the characterization of new probes that detect genetic losses in breast cancers from fractionated biopsies. Over 1000 products from RDA have been examined. These are first characterized by size and restriction endonuclease digestion. Unique clones are then used for Southern blotting to large panels of normal and cancer amplicons, and only those probes detecting losses only in cancers are taken for further study. This effectively (but not completely) eliminates probes that arise by loss-of-heterozygosity.

The remaining probes are then sequenced to generate PCR primers that we utilize for radiation hybrid analysis. This enables us to group probes into loci. To date we have so identified at least fifteen such loci, on chromosomes 1, 3, 4, 5, two on 8, 9, 10, two on 11, 12, 15, at least two on 17, and 21.

The locus on 3 corresponds to the FHIT locus, as already discussed. The locus on 9 corresponds to the region encoding p16, a protein involved in regulating the cell cycle that has been previously shown to be frequently lost in cancers and involved in hereditary forms of cancers. In addition, one locus maps to the locus of BRCA3, a locus known to predispose to hereditary breast cancers; one locus maps to the locus of Cowden's disease, an inherited dermatological disease which mildly elevates predisposition to breast cancer. These results encourage us to believe that our procedure effectively identifies loci of pathological significance.

Several loci appear to be repeatedly effected in breast cancers, including the loci on chromosome 3, chromosome 9 (p16), chromosome 8 (BRCA3), and chromosome 17. Overall, we detect deletions in about 70% of the samples we have examined.

In order to proceed further with our analysis we need to develop additional molecular techniques to enable us to rapidly analyse samples for frequency of deletion at a particular locus and to determine the size of deletion. We are limited in the amounts of DNA we have to analyse, and the presence of normal stroma. Thus straight forward applications of Southern blotting or PCR are problematical.

We have taken two approaches to circumvent these obstacles. First, we are developing a robust quantitative method for genomic PCR. This will enable us to examine genetic loss even in mixed populations of cancer and normal cells. Second, we are developing "amplicon hopping". This method will enable us to identify the genetic elements in an amplicon which are adjacent to a given probe in the amplicon. We can

then use these probes to blot panels of amplicons from sorted biopsies or even microdissected biopsies.

CONCLUSIONS

RDA is an effective way to identify regions of genetic change in cancers, and flow cytometry is an effective way to obtain material for analysis. Greater than one dozen loci undergoing loss in breast cancer have been identified. Some of these include loci that are commonly affected, such as the locus containing p16, and the FHIT locus on chromosome 3p. We detect abnormalities at these loci in nearly 70% of our cancer specimens. The transcriptional potential of these loci is being explored. Characterization of many other RDA probes is in progress. New technology is being developed to speed the evaluation of the importance of these candidate loci. We expect that the continued execution of our stated plan will accomplish our stated goal, the identification of tumor suppressor genes that are commonly involved in breast cancer.

REFERENCES

1. Lisitsyn, N. , Lisitsina, N. and Wigler, M. (1993) Cloning the differences between two complex genomes. *Science* 259, 946-951.

2. Lisitsyn, N., and Wigler, M. (1995) Applications of representational difference analysis to detection of genetic lesions in cancer. *Methods in Enzyomology, Oncogene Techniques* 254, 291-304.

3. Lisitsyn, N. A., Lisitsina, N. M., Dalbagni, G., Barker, P., Sanchez, C. A., Gnarra, J., Linehan, W. M., Reid, B.J. and Wigler, M.H. (1995) Comparative genomic analysis of tumors: Detection of DNA losses and amplification. *Proc. Natl Acad Sci. USA* 92, 151-155.

4. Ohta, M., Hiroshi, I., Cotticelli, M.G., Kastury, K., Baffa, R., Palazzo, J., Siprashvili, Z., Mori, M., McCue, P., Druck, T., Croce, C.M., Huebner, K. (1996) The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. *Cell* 84, 587-597.

5. Thiagalingam, S., Lisitsyn, N.A., Hamaguchi, M., Wigler, M.H., Willson, J.K.V., Markowitz, S.D., Leach, F.S., Kinzler, K.W., Vogelstein, B. (1996) Evaluation of the *FHIT* gene in colorectal Cancers. Cancer Research 56: 2936-2939.

6. Buckler, A.J., Chang, D.D., Graw, S.L., Brook, J.D., Haber, D.A., Sharp, P.A., and Housman, D.E. (1991) Exon amplification: A strategy to isolate mammalian genes based on RNA splicing. *Proc. Natl. Acad. Sci. USA* 88, 4005-4009.

PERSONNEL

Drs. Clifford Yen, Wen Wei, Mariko Nakamura, Diane Esposito, Robert Lucito and Masaaki Hamaguchi.